Measuring Surface-Induced Conformational Changes in Proteins

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Microfabricated cantilever sensors were used to measure the surface stress induced by protein adsorption onto a gold surface. Two proteins, immoglobulin G (IgG) and albumin (BSA), were studied. The change of surface stress upon adsorption of IgG was found to be compressive, whereas that of BSA was tensile. This difference is elucidated in terms of protein deformation and packing. Most stress change occurs not on adsorption but over very long time scales, up to 12 h, as protein conformational changes occur. The ability to monitor slow protein changes (e.g., from protein denaturing) is a particular strength of the technique.

There is considerable interest in protein adsorption on solid surfaces because of its importance in a wide range of biomedical and industrial applications such as in the manufacture of medical devices, drug delivery, and food processing. For example, the interaction of artificial solid surfaces (e.g., heart valves, kidney implants, or contact lenses) with surrounding fluids leads to protein adsorption which profoundly influences the subsequent interfacial events, such as blood coagulation or the interaction of the surfaces with cells and tissues. Protein adsorption on solid surfaces is a complex phenomenon and involves many dynamic steps from the initial attachment of the protein on the surface to the equilibrium conformational and orientational rearrangement of the adsorbed proteins.[1−3] The kinetics of adsorption of proteins have thus been actively studied using various methods[4−9] which provide a measurement of the rate of adsorption but give little information on the rearrangement undergone by the adsorbed biomolecules.

This Letter describes experiments undertaken to understand the adsorption of immoglobulin G (IgG) and albumin (BSA) on a gold surface in buffer solution in terms of surface stress measurements. The two proteins used are of particular interest as they are the most concentrated plasma proteins and have a significant importance in biotechnology applications. For example, home-use pregnancy tests consist of a simple assay using the antibody IgGs physisorbed on nitrocellulose and polystyrene (Unipath) and BSA is widely used as an adsorption-blocking agent in many immunoassay applications.

The measurement of the lateral forces involved in the attachment and rearrangement of macromolecules on a surface is a new and insightful experimental technique. The method is directly adapted from the studies of thin films[10] in which the bending of a cantilever is measured as a film is deposited onto one of the cantilever surfaces. Recently, there has been much renewed interest in this technique as a means to study surface stress during adsorption[11−13] because of the widespread availability of microfabricated cantilevers as used in atomic force microscopy (AFM). In this study AFM cantilevers are used for the first time to monitor surface stress changes arising from slow conformational changes in adsorbed proteins in situ, i.e., in aqueous buffer. Figure 1a shows a schematic of the general layout. The entire cantilever is immersed in a thoroughly cleaned, inert liquid cell (volume 0.3 cm^3) which contains the buffer solution. The difference of surface stress between the two surfaces of the cantilever ($\sigma_1 - \sigma_2$) can be found using Stoney's equation, which states

$$\sigma_1 - \sigma_2 = \frac{Et^2}{3(1-\nu)L^2}\Delta d \qquad (1)$$

where L is length, t is thickness, E is Young's modulus, ν is the Poisson ratio of the cantilever, and Δd is the measured deflection of the free end of the cantilever during protein deposition. The cantilever displacement is easily measured as in standard AFM by using the optical deflection of an incident laser beam. It can be shown[14] that surface stresses as small as $\sim 10^{-4}$ N/m can be detected by this method.

Equation 1 shows that the deflection of the cantilever is a function of the *difference* of the surface stresses between the top and bottom surfaces. Therefore to monitor an adsorption process using the cantilever method, one surface has to be the sensitizing surface on which the adsorption occurs and the other surface has to be inert to any adsorption process. To ensure that one of the exposed

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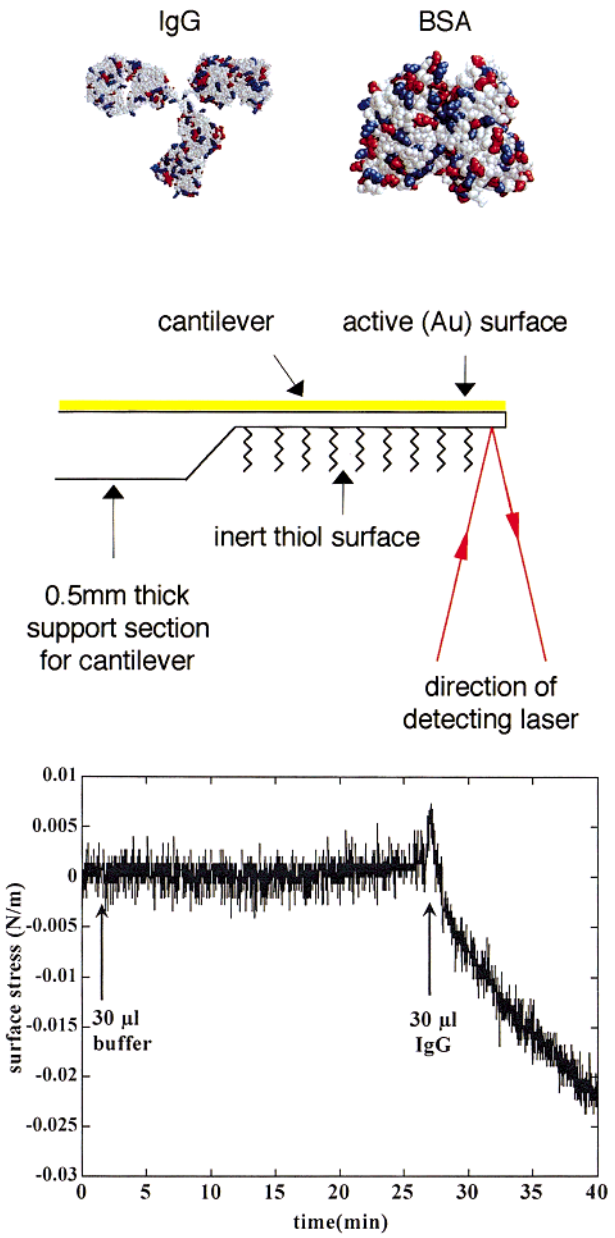

Figure 1. (a, top) Schematic of the microfabricated cantilevers used for the surface stress measurements. The approximate V-shaped Si_3N_4 cantilever dimensions are in length 200 μm, width of leg 36 μm, and thickness 0.6 μm (Digital Instruments, Santa Barbara). Their nominal spring constant is 0.12 N/m. The entire cantilever is immersed in a buffer solution (pH = 7.2, 85 g/L NaCl, 9.8 g/L Na_2HPO_4, 4 g/L NaH_2PO_4, 1 g/L NaN_3), and injected proteins adsorb on the top gold-coated surface. The bottom surface of the cantilever is coated with the thiol $HS(CH_2)_{10}(CO_2C)_2COCH_3$ (synthesized in the Department of Chemistry, Cambridge University) and is inert to protein adsorption. A laser beam is reflected off the free end of the cantilever onto a linear position sensitive detector which enables measurement of the cantilever displacement to 1 Å. Molecular models of IgG (150 000 Da) and BSA (66 000 Da).[2,21,22] The blue domains contain positively charged groups and the red domains negatively charged groups. Note that the charge is randomly distributed on the surfaces of the proteins. Hence the probability of local electrostatic forces between adsorbed proteins giving rise to surface stress is reduced. (b, bottom) Change in surface stress after first an injection of 30 μL of buffer at time = 2 min followed by an injection of 30 μL IgG at time = 28 min. This demonstrates that the buffer produces minimal surface stress over a period of 26 min as compared to the injection of IgG, which shows a large relative response.

cantilever surfaces is inert, the surface was functionalized with the thiol $HS(CH_2)_{10}(CO_2C)_2COCH_3$, which is known to impede the adsorption of a large range of proteins.[15] Subsequently, a 300 Å thick gold film was deposited on the other surface of the cantilever, to serve as the active surface. The wetting properties of the two surfaces were checked after each experiment. After protein adsorption, the active (gold) surface was always highly hydrophilic whereas the inert (thiol-functionalized) surface was distinctly hydrophobic. These observations are consistent with the bulk protein adsorption occurring solely on the active (gold) surface.

In a typical experiment 10 μL of IgG or BSA protein solution was injected into the liquid cell. The cantilever deflection following an injection was recorded for up to 11 h. The long-term signal drift is therefore a critical factor in the experiment. To minimize drift effects, the system was initially allowed to equilibrate for several hours so that the drift in the cantilever deflection was reduced to less than 10 nm/h, which corresponds to a stress of 4×10^{-3} N m^{-1} h^{-1}. As an additional safeguard, prior to an injection of proteins, an injection of the same volume of buffer was performed and always found to cause an insignificant change in surface stress.

Figure 2a shows a typical response of the cantilever exposed to a 10 μL injection of 5.85 mg/mL IgG, which corresponds to a concentration of 0.195 mg/mL. The IgG used was a mouse monoclonal antibody made against estrone-3-glucuronide and produced in hybridoma culture by Unipath Ltd., Bedford. The protein induced an overall change in surface stress which was compressive. Note that no sudden response was observed immediately after protein injection. Instead, a very slow response occurs over 11 h. This slow process is not associated with adsorption of additional protein. Surface plasmon resonance measurements of the surface coverage of IgG on gold have shown that the maximum coverage is achieved after about 10 min (R.A.B. private communication). Furthermore, a second injection of IgG 11 h following the initial injection did not produce any significant effect. Therefore, the initial IgG concentration is sufficient to provide a rapid complete monolayer coverage of the cantilever surface. It is known that various degrees of protein denaturation and cooperative effects can occur after the initial adsorption step, including very slow processes lasting several days.[1] Therefore the data of Figure 2a suggest that stress measurements can be used to monitor slow adsorption behavior rather than just the initial adsorption and immobilization processes.

What could be the origin of the slow compressive stress? In the case of IgG on gold, hydrophobic interactions dominate, and one possibility is that once adsorbed, protein unfolding can occur, i.e., for a partially hydrophobic adsorbent surface (as in these experiments, since the gold surface becomes readily contaminated with hydrophobic residues after evaporation) a denaturation of the proteins may result from the tendency to expose the hydrophobic domains, normally enclosed in the inner part of the molecule, which subsequently interact with the surface. It is reasonable to think that the protein tries to "spread" on the surface upon unfolding.[16] That is, the protein is trying to expand and therefore the gold surface is subject to a compressive stress (see Figure 2b). Alternatively, a compressive surface stress may arise if lateral forces act

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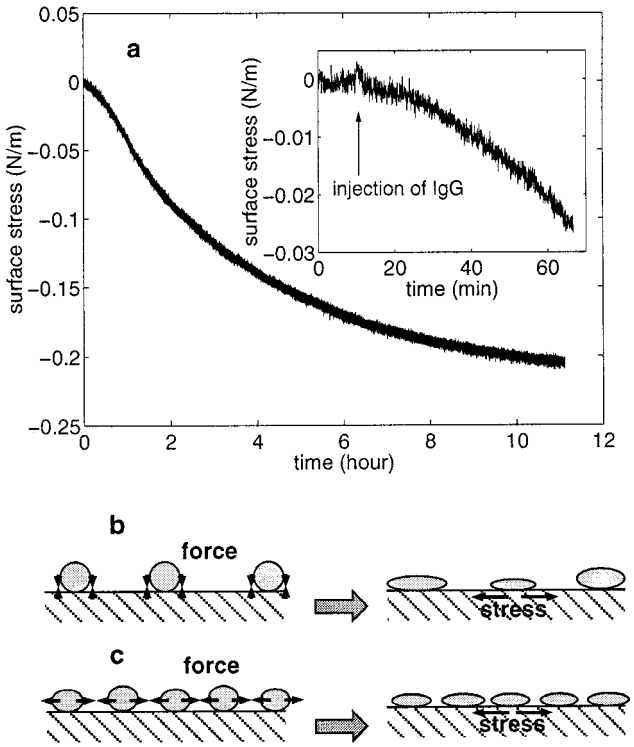

Figure 2. (a) The change in surface stress induced by a 10 μL injection of IgG on a gold surface. The inset shows the response immediately after IgG injection. Note that the injection causes minimal disturbance. The experimental drift was subtracted (5×10^{-5} N m^{-1} min^{-1}) from the response shown in the inset in order to observe the initial change of slope due to the adsorption of proteins. The change in surface stress is compressive. (b) Schematic diagram showing how protein−surface interactions may cause the proteins to unfold. The proteins try to "spread" or expand on the surface, and since they are confined within the monolayer, a compressive surface stress occurs. (c) Schematic diagram showing how attractive (hydrophobic) protein−protein interactions may cause the proteins to rearrange. Since the proteins are relatively immobile within the monolayer surface, rearrangement results in deformation of the proteins (i.e., the biomolecules tend to "flatten") and a compressive surface stress.

between neighboring proteins. Here, attractive hydrophobic forces between adsorbed proteins could cause slow surface rearrangement. If the protein is relatively immobile and confined within the monolayer (desorption is extremely unlikely[4]) some deformation of the protein can be expected, i.e., the biomolecules essentially try to "flatten"[17] causing a mechanical stress to build up in the adsorbed layer (see Figure 2c). Lateral electrostatic forces between adsorbates can also be very important in generating surface stress.[18] However, in the particular case of IgG, protein−protein strong electrostatic forces seem unlikely. The charge distribution on the protein at the working pH provides some information about the tendency of the biomolecules to attract or repel each other as most of the charged amino acids reside on the exterior of the protein molecule. The isoelectric point of the IgG used is at pH 6−7.3. At the working pH of 7.12 the net magnitude of positive and negative charges on the surface of the proteins is approximately equal, and it is therefore reasonable to assume any charge−charge repulsion between the adsorbed biomolecules is minimized under these conditions of pH.

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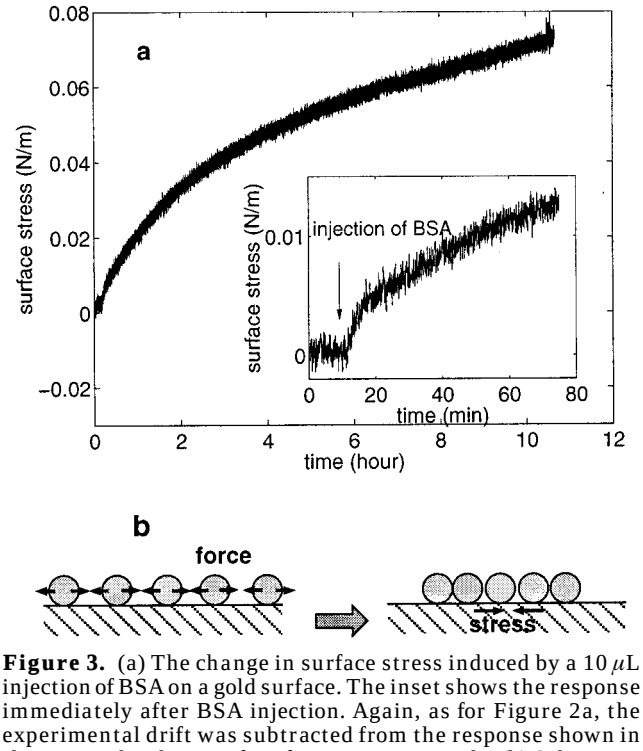

Figure 3. (a) The change in surface stress induced by a 10 μL injection of BSA on a gold surface. The inset shows the response immediately after BSA injection. Again, as for Figure 2a, the experimental drift was subtracted from the response shown in the inset. The change of surface stress is tensile. (b) Schematic diagram showing that if the proteins are relatively mobile on the surface, attractive (hydrophobic) protein−protein forces could cause the biomolecules to pack together. Since the proteins are trying to contract, a tensile surface stress results.

An interesting practical observation is that nominally identical proteins can give rise to very different stress behavior on adsorption. For example, identical experiments were performed with IgG from a second preparation batch. This batch was issued from the same cell culture as the first batch used for the data of Figure 2a but extracted and purified at a different time. The response obtained was qualitatively the same, i.e., a compressive stress change was observed, but the maximum stress change after 11 h measurement was significantly smaller, only 0.1 N m^{-1} compared to 0.22 N m^{-1}. The large difference in induced surface stress suggests that although the IgG from the two batches were nominally identical, the first batch was more inclined to undergo conformational changes at the gold surface. The experiments were reproducible and one must infer that the stress measurements reflect subtle variations in the extraction of the biomolecules from cell culture, in the plasma, or in their storage. Such slight changes in conformation behavior may relate to the observation in industrial applications that occasionally entire batches of physisorbed antibody can be inactive.

Figure 3a shows the change in surface stress induced by a 10 μL injection of 6 mg/mL BSA (Sigma, A-2153). As in the IgG experiments, no change in cantilever deflection was detected if additional BSA was added 11 h after the initial injection, which shows that coverage is essentially complete after the first exposure to BSA. As in the IgG experiments very slow surface stress changes occur. However the nature of the BSA response is entirely different from the experiments with IgG because the sign of the stress change is tensile.

What could be the origin of the tensile stress? Since the surface is uncharged, hydrophobic attraction dominates between the gold surface and the hydrophobic core of a protein. If the proteins are relatively immobile, this should

always cause a compressive stress as the protein tries to expand on the surface. It is difficult to imagine the protein contact with the surface decreasing in area upon unfolding, giving rise to a tensile stress as the protein tries to contract. Therefore the most plausible explanation for tensile stress is if the surface−protein interaction is weak, i.e., the proteins have some mobility on the surface. Hence attractive hydrophobic interactions may cause the molecules to pack together, i.e., the adsorbed proteins are trying to contract resulting in a tensile surface stress (see Figure 3b). This possibility is strengthened by the observation using fluorescence techniques of the relatively high mobility of BSA molecules on polymer surfaces.[19]

Microcantilever-based surface stress measurements provide a sensitive tool to probe the adsorption of proteins on solid surfaces, particularly over long time scales. As such, the method increases the scope for fundamental studies of the long-term behavior and conformational changes of proteins at the solid interface. Such knowledge is of critical importance in biotechnology applications involving protein-functionalized surfaces and is not available using techniques which can only measure surface coverage. A quantitative description of the origin of the changes in surface stress on adsorption and how this can be related to the microscopic, conformational changes occurring in the proteins is as yet not available and remains a difficult problem even in much simpler adsorption systems.[20]

Moreover the high sensitivity enables differences in surface stress to be observed for adsorbed proteins which are nominally identical but have undergone slightly different preparation stages. That is, the surface stress appears to be sensitive to either (i) small alterations between proteins of nominally identical structure, resulting in a different propensity for undergoing conformational changes once adsorbed or (ii) small fractions of impurities which interact with varying degree with the adsorbent surface. The stress measurements cannot distinguish between the two possible explanations, but in either case such effects are undetectable by other common adsorption monitoring techniques.

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